Wnt/β-catenin small molecule inhibitor CWP232228 preferentially inhibits the growth of breast cancer stem-like cells

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Abstract

Breast cancer stem cells (BCSC) are resistant to conventional chemotherapy and radiation, which may destroy tumor masses but not all BCSC that can mediate relapses. In the present study, we showed that the level of Wnt/β-catenin signaling in BCSC is relatively higher than in bulk tumor cells, contributing to a relatively higher level of therapeutic resistance. We designed a highly potent small molecule inhibitor, CWP232228, which antagonizes binding of β-catenin to TCF in the nucleus. Notably, although CWP232228 inhibited the growth of both BCSC and bulk tumor cells by inhibiting β-catenin-mediated transcription, BCSC exhibited greater growth inhibition than bulk tumor cells. We also documented evidence of greater insulin-like growth factor-I (IGF-I) expression by BCSC than by bulk tumor cells, and that CWP232228 attenuated IGF-I mediated BCSC functions. These results suggested that the inhibitory effect of CWP232228 on BCSC growth might achieved through the disruption of IGF-I activity. Taken together, our findings indicate that CWP232228 offers a candidate therapeutic agent for breast cancer that preferentially targets BCSC as well as bulk tumor cells.
**Introduction**

Breast cancer is the most frequently diagnosed cancer among women in the Western world (1). Conventional therapies, such as surgery, chemotherapy, and radiation therapy, have limited effects on the high rate of breast cancer recurrence (2). Recently, it has been suggested that a subset of tumor cells, called cancer stem cells (CSCs) or tumor-initiating cells, contribute to tumor growth, metastasis, and recurrence (3). Importantly, CSCs are resistant to conventional treatments, such as chemotherapy (4) and radiation (5). CSCs have been identified in almost all cancer types, including colon cancer (6), leukemia (7), and breast cancer (8). Therefore, therapeutic strategies that selectively target BCSCs will ultimately improve breast cancer treatments.

Wnt proteins are a large family of secreted, cysteine-rich molecules that play a critical role in the development of various organisms (9). The dysfunction of the Wnt/β-catenin signaling pathway has recently been implicated in several types of human cancers, including ovarian (10), colon (11), and breast cancer (12). Interestingly, accumulating evidence has revealed a critical role for Wnt/β-catenin signaling in CSCs (13). For example, mammary stem cells with high levels of Wnt/β-catenin signaling exhibit greater tumorigenic potential than their counterparts with low Wnt/β-catenin signaling (14). Therefore, these studies suggest that Wnt/β-catenin signaling is a promising target for treating breast cancer through inhibiting BCSCs.

Functional Wnt signaling activities require an interaction between β-catenin and TCF (15). The aberrant activation or transcriptional activity of β-catenin has been associated with breast stem cell amplification and tumorigenesis in a number of studies (16), suggesting that targeting the β-catenin/TCF protein-protein interaction, rather than other Wnt/β-catenin signaling components, could be an effectively target BCSCs. However, as β-catenin is an intracellular signaling molecule with no discernible enzymatic activity, this protein represents an ‘undruggable’ target (17). Recent studies have demonstrated that small molecules, including both synthetic and natural compounds, inhibit Wnt/β-catenin signaling in various cancers through the direct targeting of β-catenin. Although recently developed synthetic inhibitors targeting β-catenin, such as XAV939 (18) and IWP-2 (19), effectively
inhibit the Wnt/β-catenin pathway under in vitro culture systems, the poor pharmacokinetic and pharmacodynamic (PK/PD) profiles of these molecules have prevented in vivo preclinical investigations. Therefore, the development of inhibitors that target β-catenin and exhibit better in vivo PK/PD profiles is needed. Herein, we designed a highly potent, selective small molecule inhibitor, namely CWP232228 (U.S. Patent 8,101,751 B2), which antagonizes the binding of β-catenin to the TCF protein in the nucleus and specifically down-regulates a subset of Wnt/β-catenin-responsive genes. In vitro and in vivo studies revealed that CWP232228 suppresses tumor formation and metastasis without toxicity through the inhibition of the growth of BCSCs and bulk tumor cells. The dysregulation of insulin-like growth factor-I (IGF-I) signaling in primary breast cancers has been associated with radio-resistance and tumor recurrence (20). Although IGF-I is important for the development of breast cancer, the role of this protein in BCSCs remains unclear. We also demonstrated, for the first time, the attenuation of IGF-I-mediated BCSC sphere formation mediated by CWP232228. Taken together, these results suggest that targeting β-catenin-mediated transcription using CWP232228 has significant therapeutic potential for the treatment of breast cancer.
Materials and Methods

Cell culture and reagents

The breast cancer cell lines 4T1 and 67NR were kindly provided by Dr. Wakefield, National Cancer Institute, Bethesda, MD. Human breast carcinoma cell lines MCF7, MDA-MB-435 and MDA-MB-231 were obtained from American Type Culture Collection (Manassas, VA). Human breast carcinoma cell line Hs578t was obtained from the Korean Cell Line Bank (Seoul, Korea). Murine mammary cancer cell line 4T1 and 67NR (21) and human mammary carcinoma cell line MDA-MB-435, MDA-MB-231, MCF-7, Hs578T (21) were cultured in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin (Lonza, Basel, Switzerland) at 37°C and 5% CO2. CW232228 is designed by JW Pharmaceutical Corporation (Seoul, Korea). Docetaxel (D-1000) and recombinant IGF-I (#250-19) were purchased from LC Laboratory (Woburn, MA) and PeproTech (Rochky Hill, NJ), respectively.

Tumorsphere formation

Single cells were resuspended in serum-free DMEM (Invitrogen) containing B27 (Invitrogen), 20 ng/ml EGF, 20 ng/ml bFGF (PeproTech) and 4 μg/ml heparin (Sigma-Aldrich). Primary tumorspheres were derived by plating 50,000-200,000 single cells/well into six-well ultra-low attachment dishes (Corning, Lowell, MA). Secondary tumorspheres were plated at 50,000 cells/well. Dishes were cultivated for 7 days to enumeration of spheres. Individual spheres ≥100 μm from each replicate well (n≥9 wells) were counted under an inverted microscope at 50X magnification using the Image-Pro Plus program (Media Cybernetics). The percentage of cells capable of forming spheres, termed the ‘tumorsphere formation efficiency (TSFE)’, was calculated as follows: [(number of sphere formed/number of single cells plated) X 100].

Cell proliferation (Cytotoxicity) assay

4T1 and MDA-MB-435 cell lines were seeded in 96-well plates. Cells were treated with increasing
concentrations of CWP232228. After 48 h of incubation, cell viability was assessed by cell counting kit-8 (CCK-8, Rockville, Maryland) according to the manufacturer’s instruction. The numbers of viable cells were measured at a wavelength of 450 nm using Versamax microplate reader.

**Real-time PCR, stem cell PCR array, and Wnt signaling pathway PCR array**

Total RNA was extracted using TRIzol reagent (Invitrogen). RNA purity was verified by measuring 260/280 absorbance ratio. The first strand of cDNA was synthesized with 2 mg of total RNA using SuperScript II (Invitrogen), and one-tenth of the cDNA was used for each PCR mixture containing Express SYBR-Green qPCR Supermix (BioPrince, Seoul, Korea). Real-time PCR was performed using a Rotor-Gene Q (Qiagen, Hilden, Germany). The reaction was subjected to 40-cycle amplification at 95°C for 20 sec, at 60°C for 20 sec and at 72°C for 25 sec. Relative mRNA expression of selected genes was normalized to HPRT and quantified using the DDCT method. The sequences of the PCR primers are listed in Supplementary table 1. The stem cell PCR array (SABiosciences, Frederick, MD) and Wnt signaling pathway PCR array (SABiosciences, Frederick, MD) were performed in triplicate according to the manufacturer’s instructions.

**Flow cytometry**

FACS analysis and cell sorting were performed using FACS Calibur and FACS Aria machines (Becton Dickinson, Palo Alto, CA), respectively. FACS data were analyzed using Flowjo software (Tree Star, Ashland, OR). Antibodies to the following proteins were used: PE-conjugated Sca-1 (dilution 1/20), CD44 (dilution 1/40), CD24 (dilution 1/40), CD61 (dilution 1/40), CD133 (dilution 1/40), and LEF1(dilution 1/40). The FACS gates were established by staining with isotype antibody or secondary antibody. APC-conjugated rabbit IgG antibodies (dilution, 1/500, Invitrogen) was used as the secondary antibody to visualize LEF1 protein expression. The Aldefluor kit (Stem Cell Technologies, Vancouver, Canada) was used to isolate the population with a high ALDH enzymatic activity. Cells were stained for ALDH using the Aldefluor reagent according to the manufacturer’s
instructions and analyzed on FACS Calibur. As negative control, for each sample of cell aliquot was treated with 50 μM DEAB, a specific ALDH inhibitor. Aldefluor$^{\text{pos}}$ cells were quantified by calculating the percentage of total fluorescent cells compared with a control staining reaction. FACS Aria was used to sort Aldefluor-stained cells into Aldefluor$^{\text{neg}}$ and Aldefluor$^{\text{pos}}$ cell population.

**Luciferase reporter assay**

4T1 cells were plated at a density of $2 \times 10^4$ cells/well in 48-well plates, and transfected using Genefectine transfection reagent (Genetrone Biotech Co., Korea) according to the manufacturer’s protocol. The TopFlash (Addgene, Cambridge, MA) (22), luciferase reporter (100 ng), and Renilla luciferase thymidine kinase construct (Invitrogen) (50 ng) were used to determine luciferase activity. Luciferase activity was measured by a luminometer (Glomax, Promega, Sunnyvale, CA), using a Dual-Luciferase assay kit (Promega), according to the manufacturer’s recommendations. Total value of reporter activity in each sample was normalized to Renilla luciferase activity.

**Protein isolation and western blot analysis**

Cells were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol) containing protease inhibitor cocktail (Roche). The concentration of protein was measured with a Protein assay kit (Bio-Rad) following the manufacturer’s protocol. Total protein was subjected to SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The blot was probed with primary antibody; anti-β-catenin (Cell Signaling Technology). As a loading control, anti-β-actin antibody (Santa Cruz biotechnology) was used. Subsequently, the blots were washed in TBST (10 mM Tris-HCl, 50 mM NaCl, 0.25% Tween-20) and incubated with a horseradish peroxidase-conjugated secondary antibody. The presence of target proteins was detected using the enhanced chemiluminescence reagents (BioNote Inc., Hwaseong, Korea).

**Immunofluorescent staining**
The use of fresh breast tumor specimens was approved by the research ethic committees at the Korea National Cancer Center. Informed consent was obtained from all patients. Samples were fixed with 4% paraformaldehyde for fluorescent staining. Samples were permeabilized with 0.3 M glycine and 0.3% Triton X-100, and nonspecific binding was blocked with 2% normal swine serum (DAKO, Glostrup, Denmark). Staining was performed as described previously (23), using the primary anti-ALDH1 (Abcam, Cambridge, UK) and anti-LEF1 (Cell Signaling Technology). Alexa Fluor 488-conjugated rabbit IgG (Molecular Probes, Eugene, OR) was used to visualize ALDH1 and LEF1. Samples were examined by fluorescence microscopy (Zeiss LSM 510 Meta). The calculation of ALDH1 and LEF1 expression was based on green fluorescence area and density divided by cell number, as determined from the number of DAPI-stained nuclei, in three randomly selected fields for each specimen from a total of three independent experiments. For quantitation, an arbitrary threshold was set to distinguish specific from background staining, and this same threshold setting was applied to all the samples analyzed.

**Short hairpin RNA**

Small hairpin RNA (shRNA) targeting mouse IGF-I and non-targeting RNA were purchased from Sigma (St. Louis, MO, USA). For the efficient IGF-I shRNA transfection, reverse transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. We chose the IGF-I shRNA that is most effective in mRNA levels from five shRNA designed from the target sequence and determined by qRT-PCR and ELISA.

**Animal study**

All mice were maintained according to IACUC-approved protocols of the Lee Gil Ya Cancer and Diabetes Institute. For titration experiments, anesthetized 7-week-old female Balb/c (Orient Charles River Technology, Korea) and NOD/SCID mice (Korea research institute of bioscience and biotechnology, Korea) were inoculated with 5x10⁴ 4T1 cells and 5x10⁵ MDA-MB-435 cells into the
m.f.p in 50 µL volume (n=10 for each group), respectively. After inoculation, the mice were randomly assigned to treatment groups (i.p at CWP232228 100 mg/kg in PBS) and control group. For the combination experiment, 7-week-old female BALB/c mice were inoculated 4x10⁴ 4T1 into the m.f.p in 40 µL volume (n=10 for each group). After 10 days injection, the mice were then randomly divided into following four groups: (1) control group (2) Docetaxel (15 mg/kg, once a week) group (3) CWP228 (100 mg/kg, daily) group (4) Docetaxel + CWP group. And it was monitored for 3 weeks.

**Lung metastasis animal model**

For the metastasis and survival experiment, 9-week-old female BALB/c mice were inoculated with 5x10⁴ 4T1-Luc cells into the tail vein in 0.1 mL volume. After inoculation, the mice were randomly assigned to treatment groups (i.p at CWP232228 100mg/kg in PBS) and control group. Mice were euthanized and lungs were collected on 3 weeks, and fixed with 10 % buffered formalin. Metastasis incidence was assessed via in vivo bioluminescence measurement using an IVIS spectrum (Caliper Life Sciences). For luciferase detection, 150 mg/mL D-luciferin (Caliper Life Sciences) in PBS was injected intraperitoneally before imaging. Photometric measurement of metastasis was done by Living Image software (V. 3.1.0, Caliper Life Sciences).

**In vivo limiting dilution assay**

For the limiting dilution experiment, primary tumors were minced using scissors and incubated in DMEM (Invitrogen) mixed with collagenase/hyaluronidase (Stem cell Technologies) at 37°C for 15-20 min. Primary tumor-derived cells were inoculated into the m.f.p. of mice at varying cell densities ranging from 500 to 50000 cells in a total volume of 50 µL volume (n = 8 for each group). 4T1 and MDA-MB-435 cells-injected mice were euthanized on three and seven weeks, respectively and secondary tumors were excised for analysis. The frequency of tumor-initiating cells (TICs) was calculated using ELDA webtool (http://bioinf.wehi.edu.au/software/elda). The volume of the primary tumor was measured as previously described [33].
Statistical analysis

All the statistical data were analyzed by GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) and evaluated by two-tailed Student’s t-test. Value of P<0.05 was considered to indicate statistical significance.
Results

The stem cell markers Oct4, Sca-1, and ALDH1 are enriched in sphere-forming subpopulations

It has been suggested that three-dimensional (3D) sphere cultures of tumor cells from various cancer types, including breast (24), colon (6), brain, and pancreatic (25) cancers, have enriched cancer stem/progenitor cell populations. Recent studies have suggested that the stem cell markers Oct4 (26), Sca-1 (27), and ALDH1 (28) play important roles in maintaining the pluripotency of BCSCs. In the present study, we established a sphere-forming culture system to culture BCSCs as an *in vitro* model of breast cancer as previously described (29). To confirm whether sphere-forming subpopulations are enriched for stem cell-like properties under three-dimensional culture conditions, we examined the expression profiles of the stem cell markers Oct4, Sca-1, ALDH1, and Sox2. Consistent with previous studies, the expression levels of these markers were higher in sphere-forming cells than in cells in monolayers (Supplementary fig. 1A-C, Supplementary fig. 2B-D). We also performed FACS analysis to quantitate the percentage of the total cell population that consisted of CD44+/CD24− cells in both the monolayer and sphere cultures. As expected, the percentage of cells with this cell surface marker phenotype was markedly higher in sphere-forming cells than in monolayer cells (Supplementary fig. 1D, Supplementary fig. 2A). To determine whether sphere-forming subpopulations are enriched for stem cell-like properties under three-dimensional culture conditions, we performed an extreme limiting dilution assay (ELDA). Monolayer and sphere-forming cells were harvested and transplanted at limiting dilutions (from 50,000 to 500 cells) into mice. The repopulating unit frequency of the basal population was 1/1591 for monolayer cultures and 1/425 for sphere cultures (Supplementary table 2). Therefore, 4T1 sphere-forming subpopulations were compromised in their ability to repopulate functional BCSCs in a xenograft model, suggesting that these cells exhibit the characteristics of BCSCs and can be used to generate BCSCs in culture as an *in vitro* model for BCSC culture to evaluate the efficacy of chemotherapeutic drugs.

Enhanced expression of Wnt/β-catenin signaling-associated genes in putative BCSCs
Recently, accumulating evidence has illustrated a critical role for Wnt/β-catenin signaling in various CSCs (13). Thus, we utilized a stem cell PCR array to identify potential therapeutic targets in BCSCs (Supplementary fig. 7A). Consistent with previous studies showing elevated Wnt/β-catenin signaling activity in CSCs, the signaling array results revealed that Wnt/β-catenin signaling was activated to a greater extent in sphere-forming cells than in monolayer cells (Supplementary fig. 7B). To further assess the significance of Wnt/β-catenin signaling activity in BCSCs, we examined quantitative changes in the gene expression regulated through this pathway. The expression of downstream signaling components of the Wnt/β-catenin signaling pathway, such as Wnt1, PPAR-δ, Dvl1, LEF1, Fzd1, TCF4, and β-catenin, was markedly higher in sphere-forming cells than in monolayer cells (Fig. 1A).

Previous studies have demonstrated that aldehyde dehydrogenase 1 (ALDH1) is a marker of both normal and malignant human mammary stem cells and a predictor of poor clinical outcomes (30), and lymphoid enhancer-binding factor-1 (LEF1), is a critical regulator of Wnt/β-catenin signaling (31). Therefore, to examine the regulatory role of Wnt/β-catenin signaling in BCSCs, we analyzed the LEF1 expression levels in the ALDH-positive subpopulation. The Aldefluor-positive subpopulation showed a significantly higher level of LEF1 than the Aldefluor-negative subpopulation in multiple breast cancer cell types (Fig. 1B), suggesting that ALDH1-positive BCSC subpopulations are highly associated with enhanced Wnt/β-catenin signaling activity. To confirm whether ALDH1-positive breast cancer cells represent the LEF1-positive subpopulation, we investigated the co-expression of these markers in breast cancer cell lines (Fig. 1C), premalignant MMTV-PyMT mammary tissues (Fig. 1D; Supplementary fig. 8A), and tissue samples from breast cancer patients (Fig. 1E; Supplementary fig. 8B). As shown in Fig. 1C-E, we confirmed that the ALDH1-positive populations mostly overlapped with LEF1-positive subpopulations in both human and mouse breast cancer tissues. These results suggest that Wnt/β-catenin signaling might contribute to tumorigenic potential, representing a novel therapeutic target for eliminating BCSCs.
The β-catenin inhibitor CWP232228 suppresses BCSC growth and clonogenicity

Using a cell-based reporter system, we screened a diverse library of low molecular weight compounds for the inhibition of Wnt/β-catenin-mediated transcriptional activity. The results showed that CWP232228 was the most effective Wnt/β-catenin inhibitor (Supplementary fig. 9A). A pharmacokinetic/pharmacodynamic (PK/PD) analysis in mice indicated that CWP232228, administered i.v. at a dose of 200 mg/kg, generated an exposure of the compound in the blood at a concentration greater than 0.8 μg/ml for 7h (Supplementary fig. 9B; Supplementary. table 3). Next, we further tested the efficacy and specificity of CWP232228 to inhibit Wnt/β-catenin signaling in breast cancer cells transiently transfected with a luciferase reporter plasmid in the presence or absence of Wnt3a. In response to CWP232228 treatment, the transcriptional activity in breast cancer cells was significantly decreased in a dose-dependent manner (Fig. 2A). Using western blot analysis (Fig. 2B) and immunohistochemistry (Fig. 2C), we further investigated whether CWP232228 was sufficient to inhibit key components of Wnt/β-catenin signaling. Consistently, CWP232228 treatment significantly inhibited LEF1 expression in a dose-dependent manner. Approximate IC₅₀ values were determined using a dose-response curve. In mouse (4T1) and human (MDA-MB-435) breast cancer cell lines, the IC₅₀ values were 2 and 0.8 μM, respectively (Supplementary fig. 10).

As a functional assay, we evaluated the effect of CWP232228 on primary and secondary sphere formation. Treatment with CWP232228 resulted in the disruption of primary sphere formation of both 4T1 and MDA-MB-435 cells in a dose-dependent manner (Fig. 2D). For the secondary sphere-forming assay, treated primary spheres were collected and dissociated into single cells. The cells from treated or untreated primary spheres were re-plated on culture dishes without additional treatment. Interestingly, we observed that in the presence of CWP232228, the cells derived from primary spheres did not form secondary spheres as efficiently as the cells from untreated spheres (Fig. 2D). Moreover, we evaluated the effect of CWP232228 on the sphere formation of primary breast cancer cells obtained from four different patient samples. Prior to performing in vitro experiments, the primary breast cancer cells tested positive for cytokeratins 14 expression and negative for vimentin expression.
Treatment with CWP232228 disrupted the sphere formation of primary breast cancer cells in a dose-dependent manner (Supplementary fig. 4A-D). These findings suggest that CWP232228 was sufficient to block subsequent secondary sphere formation from primary spheres in the absence of additional treatment.

In breast carcinomas, cell populations with high levels of ALDH activity are enriched in tumorigenic stem/progenitor cells (32). Therefore, we hypothesized that CWP232228 might disrupt BCSC sphere formation by regulating ALDH activity. To test this hypothesis, we used FACS analysis to investigate the effect of CWP232228 on ALDH activity. Indeed, the treatment of 4T1 and MDA-MB-435 cells with CWP232228 for 48h decreased the size of the ALDH-positive subpopulation (Fig. 2E). In this context, we examined the expression of BCSC markers in the presence or absence of CWP232228. Consistent with our hypothesis, the expression levels of these markers, including the phenotypes Sca-1+, CD133+, CD61+, CD44+/CD24-, and side populations were significantly lower after CWP232228 treatment (Supplementary table. 4).

**CWP232228 targets chemoresistant BCSCs**

It is important to compare CWP232228 with another well-known small molecule inhibitor (FH535) that targets the β-catenin/Tcf protein-protein interactions. Because deregulated cell proliferation is a hallmark of cancer cells, the anti-proliferative effects of these two compounds were determined using the MTT assay. Overall, these data showed that CWP232228 more effectively suppressed the proliferative potential of MDA-MB-435 cells and at much lower doses than the well-known small molecule inhibitor FH535 (Supplementary fig. 5A). Normal human fibroblast cell-based dose-dependent experiments showed no marked signs of toxicity at the CWP232228 dose used in this study (Supplementary fig. 5B).

Recently, it has been suggested that BCSCs are resistant to many conventional therapeutic approaches, including chemotherapy (33) and radiotherapy (34). Thus, although traditional approaches might kill the majority of tumor cells, some of the BCSCs remain unaffected, surviving and generating new
tumors. To investigate the association between chemoresistance and Wnt/β-catenin signaling, we evaluated the available breast cancer datasets using the Oncomine dataset repository (www.oncomine.org). After specifically filtering for breast cancer datasets showing a response or nonresponse to conventional docetaxel treatment, we observed significant correlations between chemoresistance and the expression of negative (GSK3β) or positive (TCF4) regulators of Wnt/β-catenin signaling (Fig. 3A and B). Importantly, we observed that both the size of ALDH-positive populations and the sphere formation in 4T1 and MDA-MB-435 cells increased in response to conventional docetaxel treatment. However, the docetaxel-enriched ALDH-positive populations (Fig. 3C and D) and sphere formation (Fig. 3E and F) were markedly reduced after CWP232228 treatment, suggesting that CWP232228 targets BCSC populations are enriched in cells resistant to conventional chemodrugs.

**CWP232228 reduces tumor growth in a murine xenograft model**

We further investigated the in vivo efficacy of CWP232228 on tumorigenesis using a mouse xenograft model. Importantly, CWP232228 treatment (100 mg/kg, administered intraperitoneally) resulted in a significant reduction in tumor volume (Fig. 4A-C). No significant changes in mortality (Supplementary fig. 6A), body weight (Supplementary fig. 6B), hematologic values (Supplementary fig. 6C), and hemolytic potential (Supplementary fig. 6D) were observed, indicating that CWP232228-associated toxicity was minimal. No obvious clinical signs, including anorexia, salivation, diarrhea, vomiting, polyuria, anuria, and fecal changes, were observed. Additionally, no significant differences in body weight were observed in mice inoculated with cancer cells (Supplementary fig. 11). To determine whether and to what extent CWP232228 treatment affects the proportion of BCSCs in vivo, we performed FACS analysis to quantitate the percentage of the total cell population with ALDH activity in primary bulk tumors with or without CWP232228 treatment. Indeed, CWP232228 treatment led to a smaller ALDH-positive subpopulation (Fig. 4D and E). The anti-tumor effects of CWP232228 were further confirmed using serially regenerated secondary tumor
xenografts derived from primary tumor tissues without additional treatment (Fig. 4F and G). Treatment with CWP232228 significantly reduced the incidence of secondary tumors, indicating that this molecule significantly impaired the tumor initiation potential of BCSCs. We further performed an extreme limiting dilution assay (ELDA) to evaluate the inhibitory effect of CWP232228 on tumorigenesis. Following the isolation of cells from freshly digested tumor tissues, we transplanted limiting dilutions (from 50,000 to 500 cells) of the cell preparations into mice without additional CWP232228 treatment. The repopulating unit frequency of the basal population was 1/1415 and 1/1 for controls and 1/7621 and 1/1510 for CWP232228 treatment in 4T1 and MDA-MB-435 cells, respectively (Table 1). Therefore, CWP232228 did not decrease the repopulation frequency of functional BCSCs in a xenograft model.

**CWP232228 targets BCSCs, bulk tumors, and metastatic tumors**

An ideal and completely curative breast cancer treatment targets both BCSCs and bulk tumor cells to prevent recurrence. For each xenotransplant, we observed the significant inhibition of tumor growth induced through CWP232228 treatment alone or in combination with docetaxel (Fig. 5A). Docetaxel treatment alone moderately impacted tumor growth (Fig. 5A). In bulk tumors, the percentage of ALDH1-positive cells increased with conventional docetaxel treatment (Fig. 5B). However, these docetaxel-enriched ALDH-positive populations were markedly reduced after CWP232228 treatment (Fig. 5B). Moreover, we used metastatic i.v. 4T1 cell models to investigate the effects of CWP232228 on the metastasis of breast cancer. We used cells stably expressing firefly luciferase and whole-body bioluminescence to non-invasively detect intravenously injected xenografts. Lung metastasis was significantly lower in the CWP232228-treated groups than in the untreated control groups (Fig. 5C). An overall increase in the survival of animals treated with CWP232228 was also observed (Fig. 5D). These results indicated that CWP232228 treatment alone is effective against conventional docetaxel-enriched BCSC populations, bulk tumors, and metastatic tumors *in vivo.*
Suppressive effects of CWP232228 on BCSCs are achieved through the disruption of IGF-I activity

We compared the expression of downstream components of Wnt/β-catenin signaling between sphere-forming cells and monolayer cells and between non-treated and drug-treated spheres to identify potential downstream targets of CWP232228 using a Wnt/β-catenin target PCR array. We screened differentially expressed genes associated with Wnt/β-catenin signaling. Two criteria for the selection of gene expression differences were employed: a significant t-test and fold-change magnitude. Among the genes examined, the level of IGF-I mRNA was significantly enhanced (≥9-fold up-regulated) in BCSCs (Fig. 6A). Interestingly, in both Wnt/β-catenin targeting PCR array (Fig. 6B) and immunocytochemical analysis (Fig. 6C), CWP232228 treatment was correlated with decreased IGF-I levels (≥8-fold down-regulated) under sphere-forming conditions. Previous studies demonstrated that the expression of IGF-I in breast cancer tissues (35) and serum levels of this protein in breast cancer patients (36) are significantly higher than those in healthy individuals. Therefore, it is reasonable to hypothesize that CWP232228 suppresses the growth of BCSCs and bulk tumors through the disruption of IGF-I activity. Consistently, these results revealed that compared with control cells, IGF-I knockdown (Supplementary fig. 12) led to smaller ALDH-positive subpopulations (Fig. 6D) and decreased BCSC sphere formation (Fig. 6E). The stimulatory effects of IGF-I on BCSC sphere formation were successfully attenuated after CWP232228 treatment (Fig. 6F). To further evaluate the CWP232228-mediated inhibition of IGF-I secretion, we performed an ELISA to quantitate the levels of IGF-I in both monolayer and sphere cultures with or without CWP232228 treatment. Consistent with immunocytochemical results, CWP232228 treatment significantly decreased IGF-I secretion under sphere-forming conditions (Supplementary fig. 13). These results suggest that the suppressive effects of CWP232228 on BCSCs are achieved through the disruption of IGF-I activity.
Discussion

Approximately 30% to 50% of the patients diagnosed with early stage breast cancer are likely to progress to the metastatic stage, despite treatment with surgery and/or chemotherapy (37). Thus, the CSC concept has emerged as an important milestone in the understanding of chemodrug resistance and cancer recurrence (38). Based on their characteristics, targeting and eradicating CSCs represents a potential strategy for significantly improving clinical outcomes. Moreover, Fillmore et al. revealed a 30-fold increase in the BCSC population in various breast cancer cell lines after conventional chemotherapy (39). The available conventional therapeutic agents primarily eliminate the bulk of a tumor mass but do not affect BCSCs (40). Thus, the identification and development of BCSC-targeting therapeutic agents is urgent.

In recent years, a number of studies have suggested that the dysregulation of Wnt/β-catenin signaling occurs in human breast cancer (41). In this context, the high expression of β-catenin might be an important clinical and pathological feature of breast cancers and a predictor of poor overall survival (42). Mutations in the N-terminal domain of β-catenin have been observed in 92% of patients with metaplastic breast carcinoma (MBC) (43). Consistent with these findings, the results of the present study showed that the levels of Wnt/β-catenin signaling activities observed in BCSCs were significantly higher than those of bulk cancer cells, although both bulk tumor cells and BCSCs exhibit a basal level of Wnt/β-catenin signaling (Fig. 1). These results suggested that BCSCs are sensitive to therapeutic approaches targeted against Wnt/β-catenin. The β-catenin/TCF interaction is required for functional Wnt/β-catenin signaling (15); therefore, the inhibition of Wnt/β-catenin signaling through the direct targeting of β-catenin is considered an attractive therapeutic strategy. Despite academic pursuit and industrial investment, there is currently no small molecule inhibitor approved for human use. The majority of the inhibitors developed so far are in the preclinical or early clinical phase of development: XAV939 (Novartis Pharmaceuticals) and JW55 (Tocris Bioscience) are in preclinical trials, and OMP-18R5 (OncoMed Pharmaceuticals/Bayer), OMP-54F28 (OncoMed Pharmaceuticals/Bayer), PRI-724 (Prism Pharma Co, Ltd/Eisai), and LGK974 (Novartis Pharmaceuticals) are in Phase
I/II trials. If these chemical inhibitors prove to be both safe and effective for treating human cancer, then these molecules will represent powerful tools to target chemotherapy-resistant CSCs that promote metastasis.

Here, to inhibit the growth and/or self-renewal capacity of BCSCs through the suppression of β-catenin-mediated signaling, we used the small molecule inhibitor CWP232228, identified in a high-throughput screen. Follow up analyses using this compound revealed a reduction in the expression of a Wnt/β-catenin luciferase reporter and the inhibition of the expression of the Wnt/β-catenin target gene LEF1 (Fig. 2A-C). Notably, whereas the inhibition of β-catenin-mediated transcription through CWP232228 had inhibitory effects on the growth of BCSCs and bulk tumor cells, BCSCs showed a markedly greater degree of growth inhibition (Fig. 3-5). Moreover, CWP232228 treatment was sufficient to block subsequent secondary BCSC sphere formation in vitro (Fig. 2D) and secondary tumor development in the xenograft model (Table. 1), without additional treatment. These findings suggest that CWP232228 inhibits the initiation of tumor development and disrupts the physiological requirements for BCSC maintenance. The observation of markedly reduced ALDH-positive BCSC populations in bulk tumors treated with CWP232228 further supports this interpretation (Fig. 4D and E).

Breast cancer is likely to metastasize to multiple organs, primarily including the lungs, liver, and the brain. Therefore, we investigated the effect of CWP232228 on metastasis using breast cancer cell xenograft models. For this purpose, the mice were intravenously injected with mouse breast cancer cells expressing firefly luciferase (4T1-Luc), and the cells were non-invasively detected based on bioluminescence. CWP232228 significantly reduced tumor bioluminescence in a fast-growing metastatic model of mouse breast cancer. This decrease in 4T1-Luc cells reflects both the number of metastatic cancer cells (data not shown) and the tumor volume as the bioluminescent signal was measured across the whole body, integrating both optical parameters (Fig. 5C). Importantly, this efficacy was achieved without affecting primary toxicity parameters, such as mortality, body weight, hematologic values, and hemolytic potential (Supplementary fig. 6).
The aberrant activation or transcriptional activity of β-catenin has been correlated with breast stem cell amplification and tumorigenesis in a number of studies (16). Moreover, studies aiming to target CSCs have primarily focused on disrupting their self-renewal capacity rather than directly causing toxic effects; these drugs could therefore be less toxic than conventional cytotoxic chemotherapeutic drugs, as reflected by their higher than anticipated IC₅₀ values (44). This result suggests that the β-catenin/TCF interaction, rather than other Wnt/β-catenin signaling components could be an effective therapeutic target in BCSCs.

The IGF signaling pathway is one of the most important regulators of the growth, migration, and invasion of various types of cancer (45). Several in vitro experimental studies have provided substantial evidence of a role for IGF-I signaling in human breast cancer. The overexpression of the IGF-I receptor in the mouse mammary gland results in rapid mammary tumorigenesis (46). Clinical studies also support the importance of IGF-I in breast cancer. IGF-I is detected at high levels in tissue specimens (35) and serum samples (36) from breast cancer patients compared with those from healthy individuals. Moreover, the constitutive activities of IGF-I in breast cancer cells are highly associated with radioresistance and tumor recurrence (20). These studies might eventually reveal a role for IGF-I in breast malignancies with respect to tumor growth, metastatic progression, and resistance to therapy. Although the IGF-I system is important in breast cancer development, the mechanisms underlying the potential role of this protein in BCSCs remain largely unknown. Crosstalk between IGF-I and Wnt/β-catenin signaling was recently reported in colon cancer (47), oligodendroglial cells (48), and chondrocytes (49). Therefore, the interactions between these two signaling pathways in BCSCs are intriguing and need further investigation. In the present study, we also provided the first evidence that IGF-I is expressed at higher levels in BCSCs than in non-BCSCs. Furthermore, we observed an attenuating effect of CWP232228 on IGF-I-mediated functions in BCSC sphere formation and ALDH-positive BCSC populations (Fig. 6). The importance of IGF-I in the growth and/or self-renewal capacity of BCSCs is consistent with previous observations from breast cancer cell lines (50). These studies suggest that IGF-I signaling is critical for the tumorigenicity and maintenance of
BCSCs, and these cells could be selectively targeted to improve clinical outcomes through the inhibition of BCSCs.

In summary, these results provide the first demonstration that the small molecule inhibitor CWP232228 inhibits Wnt/β-catenin signaling and depletes BCSCs from bulk tumors. BCSC-depleted tumor cell populations showed diminished self-renewal capacity and decreased tumorigenicity. These findings suggest that the inhibition of Wnt/β-catenin signaling suppresses the growth and functionality of BCSCs, which might be key drivers of breast cancer metastasis and recurrence. To the best of our knowledge, the effect of CWP232228 on breast tumorigenesis has not previously been assessed. Taken together, these results suggest that by antagonizing the binding of β-catenin to the TCF protein in the nucleus, β-catenin inhibitors, specifically CWP232228, might be novel BCSC-targeting agents for the treatment of breast cancer.
Acknowledgement

This research was supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI) and the Ministry of Health & Welfare, Republic of Korea (grant number: HI11C15120000).
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Figure legends

Figure 1. Expression profiles of Wnt/β-catenin signaling-related genes in BCSCs. The mRNA expression of Wnt1, TCF4, PPAR-δ, Fzd1, β-catenin, LEF1, and Dvl1 in 4T1 monolayer and sphere-forming cells was measured using real-time PCR (A). The percentages of LEF1-positive cells in both the ALDH-positive and ALDH-negative subpopulations of various breast cancer cell types (murine mammary cancer cell line 4T1 and 67NR and human mammary carcinoma cell line MDA-MB-435, MDA-MB-231, MCF-7, Hs578T) were evaluated by flow cytometric analysis (B). The breast cancer cell lines 4T1 and MDA-MB-435 (C), premalignant MMTV-PyMT mammary tissues (kindly provided by Dr. Wakefield, National Cancer Institute, Bethesda, MD) (D), and breast cancer tissues (kindly provided by Dr. Lee, National Cancer Center, Korea) (E) were co-stained with antibodies specific for ALDH1 and LEF1. The nuclear localization of LEF1 is dependent on the cytoplasmic expression of ADLH1. DAPI staining was used to label the nuclei within each field. The results are presented as the means ± SD from three independent experiments. * P<0.05, ** P<0.01, *** P<0.001.

Figure 2. Effect of CWP232228 on the growth and clonogenicity of BCSCs. β-catenin responsive TOPFlash luciferase assays revealed that CWP232228 inhibits recombinant Wnt3a-induced Wnt/β-catenin signaling in mouse breast cancer cells (4T1). CWP232228 treatment strongly attenuated Wnt3a-induced TOPFlash activity (A). The inhibitory effect of CWP232228 on the expression of LEF1, a Wnt/β-catenin signaling target gene, was assessed in 4T1 cells through western blot analysis (B) and immunocytochemistry (C). CWP232228 inhibited primary (with CWP232228 treatment) and second sphere formation (without additional CWP232228 treatment) in both 4T1 and MDA-MB-435 cells. The sphere sizes greater than 100 µm were enumerated, and a representative image of a tumor sphere is shown. The data represent an average of three independent experiments (D). The treatment of 4T1 and MDA-MB-435 cells with CWP232228 for 48h decreased the percentage of ALDH-positive cells in the total cancer cell population (E). Abbreviations: TSFE, Tumor sphere-forming
efficiency. DAPI staining was used to label the nuclei within each field. β-actin was used as an internal control. The results are presented as the means ± SD from three independent experiments. * P<0.05, ** P<0.01, *** P<0.001. (MDA-MB-435, M-435).

**Figure 3. Effect of CWP232228 on conventional chemodrug-enriched BCSC populations.** A significant correlation between chemoresistance and the expression of a negative (GSK3β) (A) or positive (TCF4) (B) regulators of Wnt/β-catenin signaling was observed in human breast cancer datasets available through the Oncomine dataset repository (www.oncomine.org). The primary mammospheres from 4T1 and MDA-MB-435 cells treated with vehicle alone or with docetaxel (5 nM) and CWP232228 (1 µM for 4T1 cells; 0.2 µM for MDA-MB-435 cells) either alone or in combination were evaluated to determine the proportion of cells in the ALDH-positive subpopulation (C-D) and the relative numbers of sphere-forming units (E-F). Abbreviations: TSFE, Tumor sphere-forming efficiency. The results are presented as the means ± SD from three independent experiments. * P<0.05, ** P<0.01, *** P<0.001.

**Figure 4. The effects of CWP232228 on tumorigenesis in a murine xenograft model.** Schematic representation of the experimental protocol as described in the materials and methods section (A). The mice were implanted with 4T1 (5 x 10⁴ cells/mouse) and MDA-MB-435 cells (5 x 10⁵ cells/mouse) through orthotopic injection into the thoracic mammary fat pads. Tumor tissue was isolated from mice bearing 4T1 or MDA-MB-435 cell tumors that had been treated with CWP232228 (100 mg/kg, intraperitoneally) or vehicle (PBS). Tumor volumes were measured as described in the materials and methods section (B-C). The effects of CWP232228 on the ALDH-positive subpopulation as a proportion of the total cells in bulk tumors were assessed through flow cytometric analysis (D-E). 4T1 and MDA-MB-435 xenografts from mice treated with CWP232228 or vehicle were dissociated into single cell suspensions and injected into the mammary fat pads of mice in limiting dilutions (50,000, 10,000, 2,000, and 500). Tumor formation was observed for 4 weeks (4T1 cells) and 8 weeks (MDA-
MB-435 cells) following inoculation. The BCSC frequency was calculated using the extreme limiting dilution assay (ELDA) (F-G). The results are presented as the means ± SD from three independent experiments. * P<0.05, ** P<0.01, *** P<0.001.

**Figure 5. The effects of CWP232228 on BCSCs, bulk tumors, and metastatic tumors.** The mice were implanted with 4T1 cells (5 x 10⁴ cells/mouse) through orthotopic injection into the mammary fat pads. Tumor tissue was isolated from tumor-bearing mice treated with vehicle alone or with docetaxel (15 mg/kg) and CWP232228 (100 mg/kg) alone and in combination. Tumor volumes were measured as described in the materials and methods section (n=10). (A). The ALDH-positive subpopulation, as a proportion of the total cell population in bulk tumors, was assessed through immunohistochemistry (B). Monitoring tumor growth through whole-body bioluminescence imaging. Growing 4T1 cells expressing firefly luciferase were intravenously injected into mice. At one day after cancer cell injection, CWP232228 was intraperitoneally administered (100 mg/kg body weight). The mice were subjected to weekly bioluminescence imaging. Representative images at week 2 are shown (n=10). (C). The survival rate of 4T1 xenograft tumor-bearing mice following treatment with CWP232228 (100 mg/kg body weight) or vehicle (PBS) (n=12). (D). DAPI staining was used to label the nuclei within each field. The results are presented as the means ± SD from three independent experiments. * P<0.05, ** P<0.01, *** P<0.001.

**Figure 6. Antagonistic interaction between Wnt/β-catenin signaling and IGF-I in BCSC sphere formation.**

The data from a PCR array of Wnt/β-catenin signaling targets are presented as a heatmap of differentially expressed genes in 4T1 monolayer versus 4T1 sphere-forming cells (A) and in tumorspheres treated with CWP232228 (1 µM) versus tumorspheres treated with vehicle control (B); decreased (green) or increased (red) expression compared with the mean mRNA expression. The IGF-I mRNA levels are presented as fold-changes relative to controls. The inhibitory effect of
CWP232228 (1 μM) on IGF-I expression in 4T1 tumorspheres was assessed using immunocytochemistry (C). 4T1 cells transfected with IGF-I shRNA versus cells treated with control shRNA were evaluated for the ALDH-positive subpopulation as a proportion of the total cells (D) and the relative numbers of tumorsphere-forming units (E). The primary tumorspheres from 4T1 cells treated with vehicle alone or with CWP232228 (1 μM) and IGF-I (100 ng/ml), alone or in combination, were evaluated for the relative numbers of tumorsphere-forming units (F). Abbreviations: TSFE, Tumor sphere-forming efficiency. DAPI staining was used to label the nuclei within each field. The results are presented as the means ± SD from three independent experiments. * P<0.05, ** P<0.01, *** P<0.001.
**Table 1.**

<table>
<thead>
<tr>
<th>Estimated Cell Frequency</th>
<th>4T1</th>
<th>MDA-MB-435</th>
<th>P values</th>
<th>4T1</th>
<th>MDA-MB-435</th>
<th>P values</th>
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<tr>
<td>Cell No. of Inoculation, % (n)</td>
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<td>CWP232228</td>
<td>P values</td>
<td>Control</td>
<td>CWP232228</td>
<td>P values</td>
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Estimated CSC Frequency by ELDA (95% CI)

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* 4T1 and MDA-MB-435 xenograft from mice treated with CWP232228 or vehicle were dissociated into single cell suspensions and injected into the mammary fat pads of mice in limiting dilutions (50,000; 10,000; 2,000; 500). Tumor formation was observed for 4 weeks (4T1 cells) and 8 weeks (MDA-MB-435 cells) following inoculation. BCSC frequency was calculated using extreme limiting dilution assay (ELDA).
Figure 1

A

![Graph showing relative mRNA levels of different cell types.]

B

![Bar graph showing percentage of maximum expression for different cell lines.]

C

![Immunofluorescence images of breast cancer cell lines 4T1 and MDA-MB-435.]

D

![Immunofluorescence images of MMTV-PyMT mouse models.]

E

![Immunofluorescence images of breast cancer tissue.]

Expression of LEF1(%)
Figure 4

A

1st generation

Inoculation

CWP232228 Treatment

Necropsy & Injection

2nd generation

Without Additional CWP232228 Treatment

Necropsy

Breast cancer cells

ALDH^pos CSC frequency in tumor-derived cell

B

4T1 (1st generation)

<table>
<thead>
<tr>
<th>Days after inoculation</th>
<th>Control</th>
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</tr>
<tr>
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Tumor Volume (mm^3)

C

MDA-MB-435 (1st generation)

<table>
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<th>Days after inoculation</th>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
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</table>

Tumor Volume (mm^3)

D

4T1 (1st generation)

<table>
<thead>
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<th>CWP232228 (mg/kg)</th>
<th>Adefluro^pos Population (%)</th>
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</tr>
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</table>

E

MDA-MB-435 (1st generation)

<table>
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<th>CWP232228 (mg/kg)</th>
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<tbody>
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<tr>
<td>100</td>
<td>4</td>
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F

4T1 (2nd generation)

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<th>Tumor Volume (mm^3)</th>
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</thead>
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<tr>
<td>100</td>
<td>1800</td>
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</table>

G

MDA-MB-435 (2nd generation)

<table>
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<th>Tumor Volume (mm^3)</th>
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<tbody>
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<td>750</td>
</tr>
<tr>
<td>100</td>
<td>600</td>
</tr>
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</table>
Figure 6

A. Magnitude of Gene Expression

B. IGF-1 mRNA Level (Fold change)

C. CWP232228

D. Aldehyde-positive Population (%)

E. Mean TSFE

F. Mean TSFE

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Gyu-Beom Jang, In-Sun Hong, Ran-Ju Kim, et al.

Cancer Res  Published OnlineFirst February 6, 2015.

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